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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) NF1 gene mutations are the genetic basis of neurofibromatosis type I, a common genetic disorder predisposing patients to neoplasia in the peripheral nervous system and other tissues. The NF1 gene encodes a protein called neurofibromin that may negatively regulate the small G-protein Ras. Abnormal activation of Ras can cause sustained cell survival and growth in some cells (a hallmark of cancer). Aberrant Ras signaling due to a mutation in the NF1 gene is thought to contribute to the development of dermal neurofibromas and malignant peripheral nerve sheath tumors, characteristic features of neurofibromatosis type I. We have recently generated a model in which the gene encoding the adapter protein CrkL is disrupted resulting in a phenotype similar to that of NF1 disruption, including heart defects (double outlet right ventricle and ventricular septal defect), exencephaly and peripheral nerve defects. Our preliminary analysis of the CrkL mutant phenotype indicates that CrkL is essential for neural crest cells. We propose in this application to study the biological role of CrkL during development in conjunction with NF1 (Specific Aim 1) and to determine the role of the CrkL protein in regulation of Ras signaling in neural crest cells (Specific Aim 2).				
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Introduction

The Ras pathway has been a focus of research to understand how proliferation, survival, and differentiation are regulated in the cell. The Neurofibromatosis Type I gene (*NF1*) encodes a GTPase-activating protein (GAP) called neurofibromin that negatively regulates the small G-protein Ras. Therefore, unregulated Ras activity has been implicated in neurofibromatosis type I. *Crk-Like* (*Crkl*) encodes an adapter protein that links tyrosine kinase substrates to guanine nucleotide exchange factors (GEFs) for small G-proteins such as Ras. Although broadly expressed, *Crkl* is concentrated in neural crest derivatives during mouse development. Interestingly, overall phenotypes of *Nf1*^{-/-} and *Crkl*^{-/-} mouse embryos are similar. We propose to study *Crkl*^{-/-} cells and embryos in combination with *Nf1* in order to shed light onto the mechanisms by which Ras pathway modulates cell proliferation, survival, and differentiation.

Body

Specific Aim 1: To determine the developmental mechanism underlying defects in *Crkl*^{-/-} embryos and to investigate genetic interactions between *Nf1* and *Crkl* during development.

Task 1-a. Assess cell proliferation and apoptosis in neural crest derived and other tissues affected in *Crkl*^{-/-} embryos (months 1-6).

Despite an unexpected breeding problem mentioned in our previous report, we have successfully marked the neural crest derivatives by combination of *Wnt1::Cre* and *R26R* Cre reporter strains (Fig. 1). We have noted that approximately 50% of *Crkl*^{-/-} embryos show poor contributions of neural crest cells to the conotruncus of the heart as well as proximal regions of pharyngeal arches, although they can populate distal regions of arches (Fig. 1). We are determining whether poor cell proliferation and/or abnormal cell death underlies this reduced contributions of neural crest cells. This is an exciting finding that at least partly explain abnormal development of many neural crest derived tissues in *Crkl*^{-/-} embryos. As *Nf1* also plays an important role in neural crest cells, these observations warrant further investigations for potential interaction of *Crkl* and *Nf1*. This task is at near completion.

Task 1-b. Analyze chimeric animals for cell autonomous defects (months 6-18)

This task has been delayed due to the breeding problem mentioned in the annual report submitted in 2003.

Task 1-c. Analyze embryos heterozygous or homozygous for mutation in both *Nf1* and *Crkl* (months 12-18)

We have started *Nf1* and *Crkl* genetic cross. We are currently analyzing

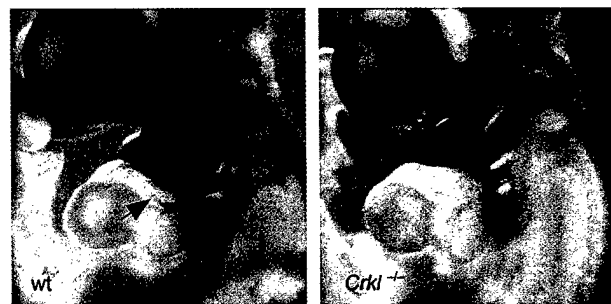


Fig. 1 Altered distribution of neural crest derivatives in *Crkl*^{-/-} mouse embryos. *Wnt1::Cre*;*R26R*/+ compound heterozygous embryo in wild type or *Crkl*^{-/-} background were stained with Salmon-gal. Embryos were obtained from *W-Cre*;*Crkl*^{-/-} X *R26R*/*R26R*; *Crkl*^{-/-} timed mating as outlined in Aim 1. No background staining was found in *R26R*/+ littermate controls without the *W-Cre* transgene (not shown). Consistent with the report from the Sucov group, neural crest cells already populate the conotruncus at this stage in wild type as pointed by the arrow. Reduced contributions of neural crest cells to craniofacial and pharyngeal arch areas are noticeable particularly in the conotruncus (arrow) and proximal regions of pharyngeal arches in *Crkl*^{-/-} embryos.

the phenotype in compound *Nf1;Crkl* mutant embryos.

Specific Aim 2: To characterize proliferation, differentiation, and survival of *Crkl*^{-/-} neural crest cells in response to neurotrophic factors and to examine Ras activity in these cells

Task 2-a. Assess cell proliferation, differentiation, and survival of *Crkl*^{-/-} neural crest cells (and wild type controls) in response to neurotrophic factors (months 18-24)

Nerve growth factor (NGF) and fibroblast growth factor (FGF) are neurotrophic factors critical for neural crest cells¹. As mentioned in the previous report, poor responses of *Crkl*^{-/-} cells to FGF makes it difficult to use neural crest cells in culture for detailed analysis (as they rely on FGF). We therefore continue to characterize impaired FGF signaling pathways in mouse embryonic fibroblasts (MEFs) obtained from wild type and *Crkl*^{-/-} embryos.

The MAP kinases Erk1 and Erk2 are known to be activated by the cascades of phosphorylation events downstream of Ras—the target of *Nf1*. In the previous annual report, we described poor Erk1/2 activation in *Crkl*^{-/-} MEFs in response to Fgf8. During the past year, a group led by Drs. J. T. Parsons and A. D. Catling (University of Virginia) reported that the MAP kinase kinase Mek1 is phosphorylated by Pak, a serine/threonine kinase, at S298 and that this phosphorylation is required for efficient activation of Mek1 by the Ras pathway². The group also showed that S298 phosphorylation is dependent on cell-matrix adhesion. We have previously shown that *Crkl* plays an important role in cell-matrix adhesion signaling to activate the small G-proteins Rac1 and Cdc42 by bridging tyrosine kinase substrate such as p130^{Cas} (aka, Bcr1 and Crkas) and Dock1 (aka Dock180)³. We therefore realize a potential role of *Crkl* in Mek1 phosphorylation at S298 since Rac1 and Cdc42 can activate Pak. Indeed, we have found that *Crkl*^{-/-} MEFs show poor S298 phosphorylation, and that re-introduction of *Crkl* transgene into these cells increases S298 phosphorylation (Fig. 2). These results (together with the results described in our previous annual report) indicate that *Crkl* participates in multiple pathways which converge upon Mek to activate the MAP kinase efficiently.

Expression of the homeobox gene *Barx1* is dependent on *Fgf8* in the neural crest-derived mesenchyme of the first

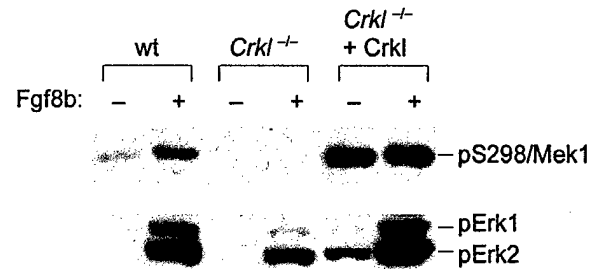


Fig. 2 *Crkl* is required for full activation of the MAP kinase kinase Mek1 and the MAP kinase Erk1/2. Phosphorylation of Mek1 at S298 was detected by S298 phospho-specific anti-Mek1 antibody. Activation of Erk1/2 was detected by T202/Y204 phospho-specific anti-Erk antibody. Cell lysates were prepared from cultured mouse embryonic fibroblasts 10 min after incubation with or without Fgf8b at a final concentration of 12.5 μ g/ml.



Fig. 3 *In situ* RNA hybridization for *Barx1* expression at E10.5. Expression of the homeobox gene *Barx1* is reduced in the pharyngeal mesenchyme in *Crkl*^{-/-} embryos. One wild type littermate and two *Crkl*^{-/-} embryos are shown. Note that *Barx1* expression in the developing stomach (st) is similar between wild type and *Crkl*^{-/-} embryos, and therefore works as an internal control for differential *Barx1* expression levels in the pharyngeal arches (1-3). vt, ventricle; ov, otic vesicle.

pharyngeal arch in mouse and chick embryos^{4,5}. In order to confirm impaired Fgf8 signaling in the absence of Crkl in vivo, we have examined Barx1 expression using in situ RNA hybridization (Fig. 3). Indeed, we have found that *Crkl*^{-/-} embryos show poor expression of *Barx1* in the neural crest-derived mesenchyme in the pharyngeal arches (Fig. 3).

Following Tasks will be initiated in the upcoming years:

Task 2-b. Assess real-time activity and signaling of small G-proteins in *Crkl*^{-/-} and wild type neural crest cells (months 24-30)

Task 2-c. Assess phenotypic rescue of defects in *Crkl*^{-/-} neural crest cells by downstream mediators of Crkl and Ras (months 24-36).

Key Research Accomplishments

We have made the following key observations:

- Neural crest cells migrate and contribute initially to pharyngeal arches in *Crkl*^{-/-} embryos. However, their final contributions to pharyngeal arches and the outflow tract are reduced.
- Crkl is essential for Fgf8 signaling, partly by mediating adhesion-dependent phosphorylation of Mek1 at S298.
- Crkl plays a role in Fgf8-induced expression of key genes such as *Barx1* in neural crest cells during development.

Reportable Outcomes

Part of our observations described in this annual report has been presented in the following research conference:

- The 10th Weinstein Cardiovascular Development Conference, held at Leiden, The Netherlands (May 13-16, 2004).
- The 10th Meeting on Protein Phosphorylation and Cell Signaling, held at the Salk Institute, La Jolla, CA (June 25-29, 2004)

Following manuscript includes our studies in which we found that Crkl can modulate Fgf8 induced signaling in vivo and in cells.

- Moon, A. M., Guris, D. L., Li, L., Miller, A. C., and **Imamoto, A.** Crkl deficiency disrupts Fgf8 signaling in the pathogenesis of *del22q11* syndromes. *Submitted* (2004).

Conclusions

We have found that Crkl dependent signaling pathways play important roles in Fgf8 and adhesion signaling pathways that regulate activation of the MAP kinase. These observations are in good agreement with our in vivo analysis of neural crest contributions and gene expression. Our current work will elucidate the function of NF1, a negative regulator of Ras. Since the Ras pathway is crucial for Fgf8 signaling and defects in Nf1 and Crkl-deficient mice are similar, we anticipate some interaction of these genes during development.

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(contains unpublished data)

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